



Membrane stabilization effect and histological changes in the heart in experimental myocardial rats with *Zanthoxylum armatum* fruit

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ABSTRACT: Membrane bound adenosine triphosphatases (ATPases) shed a massive function into the contraction and relaxation of the heart muscle via keeping the normal ion levels within the myocyte. The current study aims to assess the potency of *Zanthoxylum armatum* (*Z. armatum*) fruit on membrane bound ATPases and ions in Isoproterenol (ISO) induced myocardial infarcted rats. The hydroethanolic extract of *Z. armatum* fruit was administered at a dose of 200 and 400mg/kg body weight for 30 days to male Wistar albino rats. On 28th and 29th day, ISO (8.5mg/100g body weight) used to be administered to induce myocardial infarction (MI). ISO treated rats confirmed a significant increase in the levels of tissue sodium (Na⁺) and calcium (Ca²⁺) ions and membrane bound Ca²⁺ ATPase then Mg²⁺ ATPase activity. A significant decrease in tissue potassium (K⁺), Na⁺/K⁺ ATPase was observed which indicates membrane destabilization. Pretreatment with hydroethanolic extract of *Z. armatum* fruit to ISO induced rats significantly (p<0.05) prevented the altered membrane bound enzymes to near normal status. The findings of the present study indicate the protective effect of *Z. armatum* fruit on altered ion pumps and destabilization on the cardiac membrane into ISO induced MI rats which might also be due to the presence of phytoconstituents.

Keywords: membrane bound ATPase; electrolytes; Isoproterenol, *Z. armatum*.

1. Introduction

Myocardial damage is thought to be an essential trigger for the pathological process of coronary illness. Determination of membrane related enzyme like ATPases show the changes in the membrane under obsessive conditions. ATPases of cardiovascular cells play a vital role in the maintenance of normal ion levels with on the heart and are involved in the contraction and relaxation of cardiac muscle [1]. Na⁺/K⁺ ATPase catalyze the dynamic uptake of K⁺ and expulsion of Na⁺ to the detriment of ATP [2]. Maintenance of these ionic gradients is essential for all

cells; a low intracellular Na⁺: K⁺ ratio is imperative for enzyme activation in metabolic pathways, maintenance of cell volume, intracellular pH regulation and both secretory and absorptive function [3].

Ca²⁺ is a basic component in ordinary vascular tissue physiology. It regulates blood pressure, ion fluxes and the binding of a receptor to the membrane [4]. Calmodulin along with Ca²⁺ induce many enzymatic pathways [5]. Like Ca²⁺, magnesium (Mg²⁺) is a fundamental element in ordinary cardiovascular physiology [6]. These cations influenced the synthesis and

secretion of neurotransmitters, water, solute excretion, cardiac output and intravascular volume [7]. Membrane related activities of Mg²⁺ incorporate stabilization of Ca²⁺ channels, initiation of Ca²⁺/Mg²⁺ ATPase, and regulation of Na⁺/K⁺ ATPase activity [8].

To examine the possible protective effect of drugs on the myocardial damage from MI, a generally used model is the induction of infarction by the administration of ISO in rats. ISO, a β-adrenergic agonist is a notable inducer of myocardial necrosis and interstitial fibrosis [9]. Mohanty *et al*, [10] have demonstrated that ISO prompts myocardial necrosis characterized by expanded end-diastolic volume, end-diastolic pressure and left ventricular wall thickness. Oxidative stress has been embroiled in the pathogenesis of myocardial ischemia [11]. It discourages the sarcolemmal Ca²⁺ transport and results in the development of intracellular Ca²⁺ overload and ventricular dysfunction [12]. Ca²⁺ overcharge inside the cell is identified with the enactment of the adenylate cyclase and exhaustion of adenosine triphosphate (ATP) levels [13].

Our previous studies have shown that *Z. armatum* fruit is a potent blocker of lipid peroxide formation and scavenger of superoxide anions and hydroxyl radicals. Apart from antioxidant activities, the *Z. armatum* fruit has cardiac glycosides, phenols, flavonoids and alkaloids which may substantially aid to its cardioprotective action. Based on these observations, the present investigation was aimed to evaluate the membrane-stabilizing action of *Z. armatum* fruit by analyzing the levels of transmembrane protein activity and electrolytes in myocardial tissue. This study could be helpful in the mechanism based therapeutic effect of *Z. armatum* fruit on heart injury by ischemia.

2. Materials and Methods

1.1. Collection of plant material and preparation of extract

The plant *Z. armatum* fruit was collected from Kolli hills, India. The taxonomic identify of the plant was confirmed from the ABS Botanical Conservation, Research and Training Centre, Salem, Tamilnadu, India. (Voucher Specimen No: AUT/ECP/101). The fruits were dried at room temperature and extracted with 50% hydroethanol. Then it was filtered, dried in a rotary vacuum evaporator and used for analysis.

1.2. Animals

Male Wistar albino rats (*Rattus norvegicus*) weighing about 150-180g were obtained from animal house of PSG Institute of Medical Sciences and Research, Coimbatore, Tamil Nadu, India. They were housed in polypropylene cages under a 12:12 hr light and dark cycle at around 37°C. The rats had free access to tap water and food. They were fed on a standard pellet diet (AVM Cattle and Poultry Feeds, Coimbatore) and water *ad libitum*. The clearance of the ethical committee for experimentation on animals was obtained before the start of the experiment (Proposal No: 158/PO/bc/99/CPCSEA). The experiment was carried out according to the guidelines of the Committee approved by the Animal Ethical Committee of PSG Institute of Medical Sciences and Research, Coimbatore.

1.3. Induction of MI

ISO was used to induce MI in rats. Animals were injected subcutaneously with freshly prepared ISO in sterile normal saline at a dose of 8.5mg/100g body weight.

1.4. Experimental design

Animals were divided into six groups of six rats in each group and the grouping of animal is shown in table-1. At the end of the experimental period i.e, 12hr after the second dose of ISO injection, all the rats were sacrificed by cervical dislocation under mild chloroform anesthesia. The heart tissue was excised immediately and thoroughly washed with ice-cold physiological saline and it was used for various biochemical estimations.

Table 1 Treatment schedule

Groups	Diet/Treatment
GI-Normal control rats	Standard rat pellet for 30 days
GII- <i>Z. armatum</i> fruit treated rats	Hydroethanolic extract of <i>Z. armatum</i> fruit 400mg/kg body weight for 30 days (oral intragastric tube).
GIII- ISO treated rats	ISO (8.5 mg/kg body weight) injected subcutaneously twice at an interval of 24hr on 28 th and 29 th day.
GIV- Rats pretreated with <i>Z. armatum</i> fruit + ISO	Hydroethanolic extract of <i>Z. armatum</i> fruit (200mg/kg body weight for 30 days) + ISO (8.5 mg/100g body weight) subcutaneously

GV- Rats pretreated with <i>Z. armatum</i> fruit + ISO	twice at an interval of 24hr on 28 th and 29 th day. Hydroethanolic extract of <i>Z. armatum</i> fruit (400mg/kg body weight for 30 days) + ISO (8.5 mg/100g body weight) subcutaneously twice at an interval of 24hr on 28 th and 29 th day.	1.5.3. Assay of calcium dependent triphosphatase (Ca^{2+} ATPase) The incubation mixture contained 0.1ml of 75mM Tris-Hcl buffer pH 7.6, 0.1ml of 5mM calcium chloride, 0.1ml of 2mM ATP, 0.1ml of double distild H ₂ O and 0.1ml of tissue homogenate [17]. The contents were incubated at 37°C for 15min. the reaction was the arrested by the addition of 0.5ml of cold 10%TCA. The amount of phosphorous liberated was estimated by the method of Fiske and Subbarow [15]. The enzyme activity is expressed as μ moles of Pi liberated/min/mg/protein.
GVI- Rats pretreated with standard drug + ISO	Standard drug verapamil (1mg/kg body weight for 30 days) + ISO subcutaneously twice at an interval of 24hr on 28 th and 29 th day.	

1.5. Assay of membrane bound enzymes

1.5.1. Assay of sodium potassium dependent adenosine triphosphatase (Na^+ /K^+ ATPase)

Na^+ /K^+ ATPase transport Na^+ /K^+ against concentration gradient at the cost of ATP molecule liberating inorganic phosphate (Pi). Na^+ /K^+ ATPase activity was estimated by the method of Bonting [14]. The incubation mixture contain 1ml of Tris buffer (92mM), 0.2ml of magnesium sulphate (50mM), 0.2ml of potassium chloride (50mM), 0.2ml of sodium chloride (60mM), 0.2ml of EDTA (1mM) and 0.2ml of ATP (4Mm). Thus, the assay medium in a final volume of 2.0ml. After 10min, equilibrium at 37°C in an incubator, reaction was started by the addition of 0.1ml of tissue homogenate. The reaction mixture was incubated for 15 min and the reaction was arrested by the addition of 1ml of 10% TCA. The amount of Pi liberated was estimated by the method of Fiske and Subbarow [15]. The enzyme activity is expressed as μ moles of Pi liberated/min/mg protein.

1.5.2. Assay of magnesium dependent adenosine triphosphatase (Mg^{2+} ATPase)

The activity of Mg^{2+} ATPase was assayed by the method of Ohnishi et al [16]. The assay was initiated by the addition of 0.1ml of tissue homogenate to an incubation medium containing 0.1ml of 2mM ATP, 0.1ml of double distilled water respectively in total incubation volume of 0.5ml. the contents were incubated at 37°C for 15 min. the reaction was arrested by adding 0.5ml of cold 10% TCA. The amount of Pi liberated was estimated by the method of Fiske and Subbarow [15]. The enzyme activity is expressed as μ moles of Pi liberated/min/mg of protein.

1.6. Estimation of electrolytes in heart tissue

The concentration of Na^+ and K^+ ions in heart homogenate was estimated using commercial kits purchased from Lab-Care Diagnostics Private Limited, India. The level of Ca^{2+} ion in the heart was measured by the O-cresolphthalein complexone method using a reagent kit purchased from Span Diagnostics Limited, India. The result was expressed as nmol/mg protein.

1.7. Histopathological analysis

The heart tissue were fixed in 10% formalin, underwent a dehydration process with isopropanol for 1hr then embedded in molten paraffin wax using the tissue- embedding centre, which later cooled down to formed blocks of paraffin. Each block was trimmed then sectioned about 5 μ m by using a microtome. For the heart tissue, the specimens were stained with Haematoxylin and Eosin (H&E) dye to observe the elastic fibers which mounted with DPX for microscopic examinations.

1.8. Statistical analysis

The results were articulated as mean \pm standard deviation. Statistical analysis was carried between the experimental groups using one way analysis of variance (ANOVA) employing statistical package for social science (SPSS Version 16.0). Post hoc testing was performed for inter-group comparisons using Fisher's least significant difference (LSD) tests. The level of significance was set as ($p < 0.05$).

3. Results and Discussion

The activity of Na⁺/K⁺ ATPase significantly decreased and the activities of Ca²⁺ ATPase and Mg²⁺ ATPase significantly increased in the heart tissue of the ISO induced rats (Gropu-III), compared to the normal control rats (Group-I). Oral pretreatment with *Z. armatum* fruit (200 and 400mg/kg) for a period of 30 days significantly regulated the activities of Na⁺/K⁺ ATPase, Ca²⁺ ATPase and Mg²⁺ATPase in the heart of MI induced rats. There was no significant difference between standard drug treated rats (Group-VI) and *Z. armatum* fruit treated rats at a dose of 400mg/kg (Group-V). When comparing the control rats and plant alone treated rats (Group-II) did not show any significant effect and is given in table-2.

ATPases are integral membrane proteins which require thiol groups and phospholipids (PL) to keep up their structure and function [18]. Na⁺/K⁺ ATPase, a widely considered individual from the P-type ATPase family, is associated with the regulation of cell volume, development of membrane potential and transport of supplements in tissues [19]. Na⁺/K⁺ ATPase is a lipid dependent enzyme contains (-SH) group. Mg²⁺ ATPase plays the main role in Ca²⁺ influx and cell bursting [20].

Oxidants are known to initiate lipid peroxidation and accordingly demolish PL which is required for the typical action of membrane bound protein.

Table 2 Effect of *Z. armatum* fruit on membrane bound ATPase in heart tissue homogenate of normal and ISO induced MI rats.

GROUPS	TISSUE (µmol Pi liberated/ min/ mg of protein)		
	Na ⁺ K ⁺ ATPase	Ca ²⁺ ATPase	Mg ²⁺ ATPase
GROUP I	0.59 ± 0.28	1.39 ± 0.19	6.24 ± 0.27
GROUP II	0.52 ± 0.31	1.28 ± 0.05	6.31 ± 0.14
GROUP III	0.26 ± 0.03 ^a	2.73 ± 0.09 ^a	9.21 ± 0.31 ^a
GROUP IV	0.31 ± 0.02 ^{a,b,c}	2.12 ± 0.08 ^{a,b,c}	8.31 ± 0.17 ^{a,b,c}
GROUP V	0.49 ± 0.26 ^{a,b}	1.62 ± 0.02 ^b	7.05 ± 0.04 ^{a,b}
GROUP VI	0.56 ± 0.02 ^b	1.47 ± 0.17 ^b	6.92 ± 0.12 ^{a,b}

All the values are mean ± SD of six samples in each group. a,b,c- significant at 5% level (p<0.05). Group comparison: a- GI vs GII, GIII, GIV, GV, GVI: b- GIII vs GIV,GV,GVI: c- GVI vs GIV, GV.

Table 3 Effect of *Z. armatum* fruit on level of electrolytes in heart tissue homogenate of normal and ISO induced MI rats

GROUPS	TISSUE (nmol /mg of protein)		
	Na ⁺	K ⁺	Ca ²⁺
GROUP I	5.17 ± 0.7	6.21 ± 0.57	9.79 ± 0.08
GROUP II	5.21 ± 0.02	6.14 ± 0.04	9.67 ± 0.25
GROUP III	10.18 ± 0.31 ^a	2.42 ± 0.37 ^a	16.25 ± 0.79 ^a
GROUP IV	6.24 ± 0.28 ^{a,b,c}	5.07 ± 0.74 ^{a,b,c}	11.21 ± 0.38 ^{a,b,c}
GROUP V	5.91 ± 0.12 ^{a,b}	5.64 ± 0.14 ^{a,b}	10.51 ± 0.63 ^b
GROUP VI	5.52 ± 0.71 ^{a,b}	5.86 ± 0.28 ^{a,b}	10.11 ± 0.27 ^b

Values are mean ± SD of six samples in each group. a,b,c- significant at 5% level (p<0.05). Group comparison: a- GI vs GII, GIII, GIV, GV, GVI: b- GIII vs GIV, GV, GVI: c- GVI vs GIV, GV.

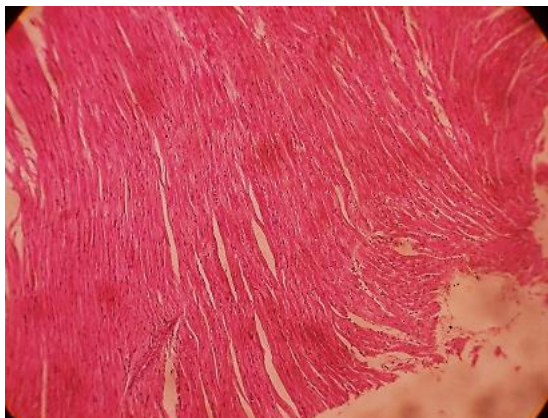


Plate 1: GI –Normal myocardial

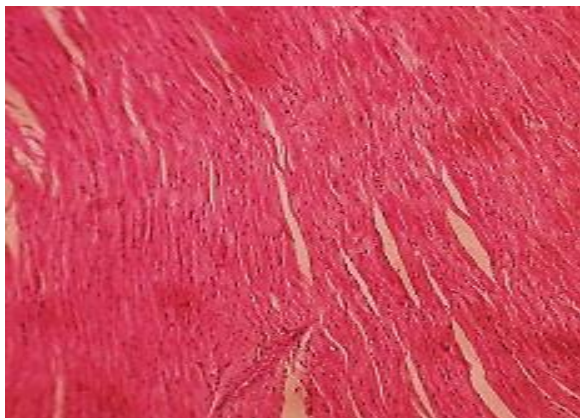


Plate 2: GII –Normal myocardial

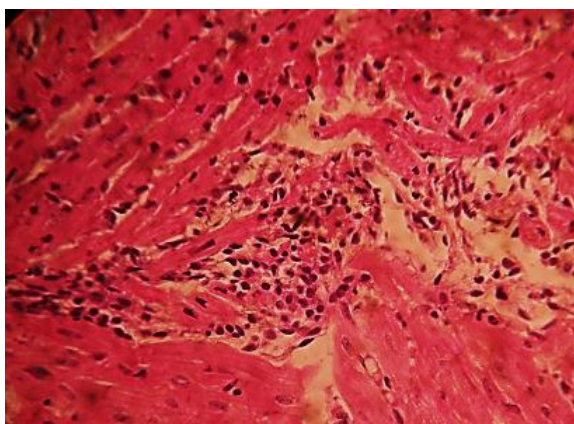


Plate 3: GIII – Focal areas of necrosis

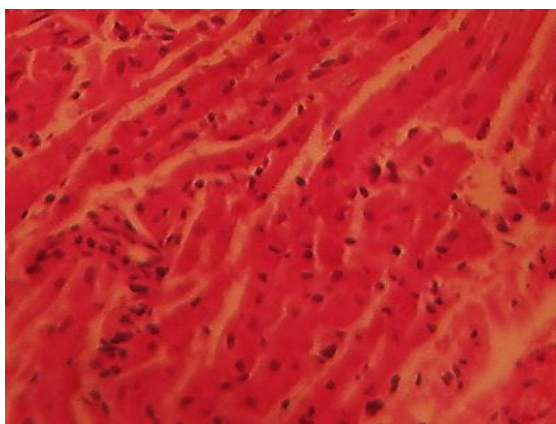


Plate 4: GIII – Myocardial tissue with mild inflammation and focal myonecrosis

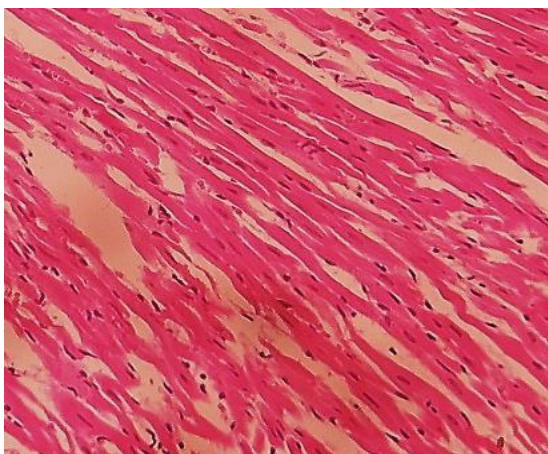


Plate 5: GIV- Normal cardiac fibres without necrosis

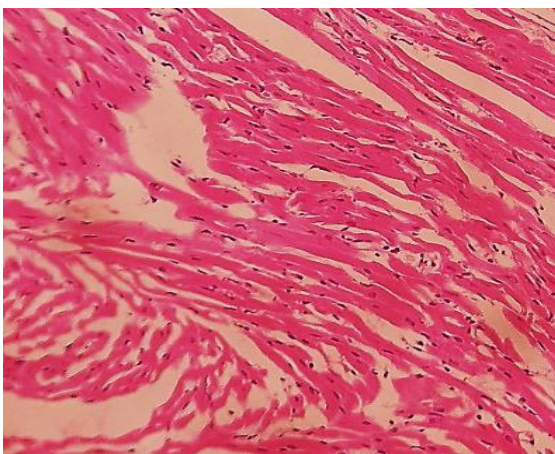


Plate 6: GVI – Cardiac fibres showing lesser inflammation and focal myonecrosis

Figure1. Histopathological observation of the heart.

ISO is a non-competitive β -adrenergic stimulator and administration of ISO is known to produce reactive oxygen species (ROS), which changes membrane PL and proteins, promoting lipid peroxidation, oxidation of thiol groups and phosphorylates cyclic adenosine monophosphate (cAMP) at numerous sites on the C-terminal chains of the Ca^{2+} ATPase [21,22,23]. Inactivation of the lipid-dependent membrane bound ATPases, increased lipid peroxidation and diminished PL content in the myocardium could be the reason behind the changes in ATPase activity.

Pre-treatment with hydroethanolic extract of *Z. armatum* fruit significantly increased the activity of Na^+/K^+ ATPase in the heart homogenate of ISO induced MI rats. The restoration of ATPase activity could be because of the blockage of ISO induced Ca^{2+} influx, keep up the membrane integrity by producing thiol group substances and scavenging the free radical in the myocardium from excess damage.

Table 3 represents the effect of the hydroethanolic extract of *Z. armatum* fruit on electrolytes in the heart tissue of normal and ISO induced rats. Rats induced with ISO, showed a significant increase in tissue Na^+ and Ca^{2+} ions with a subsequent decrease in K^+ ion when compared with normal rats. Pretreatment with hydroethanolic extract of *Z. armatum* fruit (200 and 400 mg/kg) for a period of 30 days significantly reverted this alteration in heart tissue of ISO induced rats. In standard verapamil group and *Z. armatum* alone treated group there is no significant difference in the level of electrolytes as compared to control groups.

In the cell, ATPases are personally connected with the plasma membrane and participate in the energy-dependent transport of Na^+ , K^+ and Ca^{2+} translocation [24]. An increase in Na^+ and Ca^{2+} along with the decrease in K^+ was seen in ISO injected rats. ISO administration triggers lipolysis and generation of ROS which destabilize the myocardial membrane [25]. Modification in ATPase activity results in the alteration of electrolytes concentration. The changes in the levels of Na^+ and K^+ in ISO induced rats might be attributable to loss of cellular integrity, inhibition of the Na^+/K^+ ATPase function because of energy depletion and changes in the proportion of intracellular -to-extracellular volume that indicate the severity of degenerative heart infection [26,27].

Pretreatment with hydroethanolic extract of *Z. armatum* fruit significantly ($p < 0.05$) prevented the ISO induced alteration in Na^+ , K^+ and Ca^{2+} ion levels in the heart tissue. The transport of Na^+ and K^+ amongst intra and extracellular pools and the maintenance of the transmembrane gradients are essential to cell function and integrity. *Z. armatum* fruit sustained the membrane bound ATPases which are essential to control the electrolyte levels in ISO induced MI rats.

Figure-1 shows the histology of heart tissue of normal and experimental groups of rats. The histological examinations of the heart tissue of normal rats receiving *Z. armatum* (Group II) alone did not show any significant changes when compared with that of normal control rats, showing that it does not per se have any adverse effects (Plate 2). ISO administered group (Plate 3), sections revealed focal lesions consisting of fragmentation of muscle fibres with confluent retrogressive lesions, hyaline necrosis, and sequestering mucoid edema. Also in some cases occurrence of cellular hyperplasia and central necrosis in portal areas were noted. This pathological aberration is probably related to a decline in oxygen supply with paramount rise in wall-stress.

Further, a minimal damage was observed in group IV *Z. armatum* fruit (200mg/kg) and verapamil treated rats (Group VI) after ISO administration (Plate 4,6). Histology of the heart tissue sections of Group V (400mg/kg) *Z. armatum* fruit administered rats (Plate 5) showed normal architecture of myofibrillar striations, branched appearance and continuity with adjacent myofibrils compared to the altered cardiac architecture of Group III ISO injected animals. The morphology of cardiac muscle fibers was well preserved and found to be comparable to that of normal control rats, indicating the cytoprotective action of *Z. armatum* fruit.

4. Conclusion

Membrane proteins are considered as an extremely potent drug targets because of their role transporters and mediators in the interaction of cells with the surrounding environment. The failure of the cell membrane to maintain normal transmembrane ionic distribution through ion pumps is thought to be a major event in the pathogenesis of ischemia and arrhythmia. Our results conclude that *Z. armatum* fruit is highly effective in

preventing cardiac dysfunction in rats, conceivably due to membrane stabilizing the property by maintain the levels of electrolytes and ATPases and cytoprotective activity by maintain the normal architecture of heart tissue.

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Conflict of interest:

There are no conflicts of interest.

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Competing Interests:

The authors declare that they have no competing interests.